EFFECT OF STORAGE STABILITY ON THE ANTIMICROBIAL ACTIVITY OF MICROENCAPSULATED VIA SPOUTED-BED ROSEMARY EXTRACTS

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ABSTRACT

Long-term stability of microencapsulated via spouted-bed rosemary hydro-alcoholic extracts was studied regarding their antibacterial activity. The samples were stored in dark place at 4°C for 10 months. Five batches with variation in the incorporated extracts and composition of the coating agent were tested by means of the agar diffusion method against two model strains, the Gram-negative Escherichia coli K12 and the Gram-positive Bacillus subtilis 356. Inhibitory effect was obtained from all five samples against the Gram-positive bacteria. None of the studied products showed activity against the Gram-negative strain. The most prominent effect against B. subtilis 356 was detected by microcapsules of batch 5 containing the highest amount of fresh rosemary extract, followed by the samples with concentrated extract (batch 2 and 4). The least inhibitory effect was observed by the microcapsules of batch 1 and 3, containing a lower quantity of fresh non-concentrated extract.

Keywords: hydro-alcoholic extracts, microencapsulated rosemary extracts, storage stability, antibacterial activity, agar diffusion method.

INTRODUCTION

Rosmarinus officinalis L. (rosemary) is a well-known herb used widely for flavoring foods, perfume production, cleaning products and shampoos [1, 2]. Recently the interest in this spice has been increased due to its wide-ranging antimicrobial, anti-inflammatory and antioxidant properties [3]. The ability of rosemary oil to protect foods against pathogenic and spoilage microorganisms has been previously reported [3, 4] and the inhibitory effect has been found to be a result of the action of rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol and isorosmanol. They interact with the cell membrane, causing changes in genetic material and nutrients, changing the transport of electrons, leakage of cellular components and changes in fatty acid production [5]. In general, a higher antimicrobial activity of essential oils is observed on Gram-positive bacteria than Gram-negative bacteria [6]. This is due to the lipophilic ends of lipoteichoic acids in cell membrane of Gram-positive bacteria which simplifies the penetration of hydrophobic compounds of essential oils [7]. On the other hand the protecting role of outer membrane proteins or cell wall lipopolysaccharides of Gram-negative bacteria limits the diffusion rate of hydrophobic compounds through the lipopolysaccharide layer [8].

Unfortunately, there are difficulties when using bioactive compounds such as poor long-term stability; they are easily affected by pH variation, presence of light, oxygen, etc. [9]. In this sense, the use of microencapsulation techniques is emerging as a way to ensure longer storage life for the bioactive compounds. Microencapsulated forms of plant extracts are easier applicable in food preservation and active packaging. There are many researchers whose work is focused on

different ways for microencapsulating plant extracts [10 - 15]. Donsì et al., 2011, performed antibacterial experiments with encapsulated terpenes mixture from *Melaleuca alternifolia* and D-limonene. They observed excellent protection against degradation or evaporation without sacrificing the antimicrobial activity [16]. Still reports on encapsulation of rosmarinic acid or rosemary extracts rich in phenolic compounds are scarce. Hence, there is a lack of data on the storage stability of such microencapsulated extracts and in particular their antibacterial activity.

The aim of the present work is to evaluate the antibacterial activity of microencapsulated via spouted-bed rosemary hydro-alcoholic extracts against two bacterial strains after 10 months storage thus demonstrating their applicability as functional food ingredients and in active packaging materials.

EXPERIMENTAL

Materials

Liquid and solid (agar) medium, Luria Bertani (LB) for *E. coli* K12 and Nutriant Broth (NB) for *B. subtilis* 356, both from HiMedia Laboratories were prepared for the bacteria. The strains were obtained from the Bulgarian National Bank of Industrial Microorganisms and Cell Culture and conserved in our laboratory. The cultures were incubated in Shaker ES-20/60. Sterile filter paper discs (6 mm in diameter) by HiMedia Laboratories were used for the antibacterial experiments.

Rosemary extracts preparation

Microencapsulated rosemary extracts used in this work were produced via spouted-bed processing of fresh and concentrated rosemary extracts obtained with 37 mass % ethanol in water as a solvent for extraction. Both fresh extracts and their concentrates resulting from vacuum evaporation were employed in the coating solution for spouted bed microencapsulation. Detailed description of the solvent extraction and microencapsulation methodologies can be found elsewhere together with the composition of the coating solution for each of the five microencapsulated products (Fig. 1A) [17].

After the microencapsulated rosemary extracts were obtained, part of each batch was stored in a sterile container in darkness at 4°C for 10 months (Fig. 1B). The microcapsules resulted physically stable with no visible change in the consistency and color of the particles (Fig. 2).

Antibacterial experiments

The antibacterial activity of microencapsulated rosemary extracts after long-term storage was tested against facultative anaerobic Gram-negative *E. coli* K12 and aerobic Gram-positive *B. subtilis* 356 by the use of the agar diffusion test.

The cultures were grown, sub-cultured and maintained in LB and NB solid medium and stored in the fridge at 4°C. For the experiments a single colony of the organisms was inoculated into 50 ml LB and NB

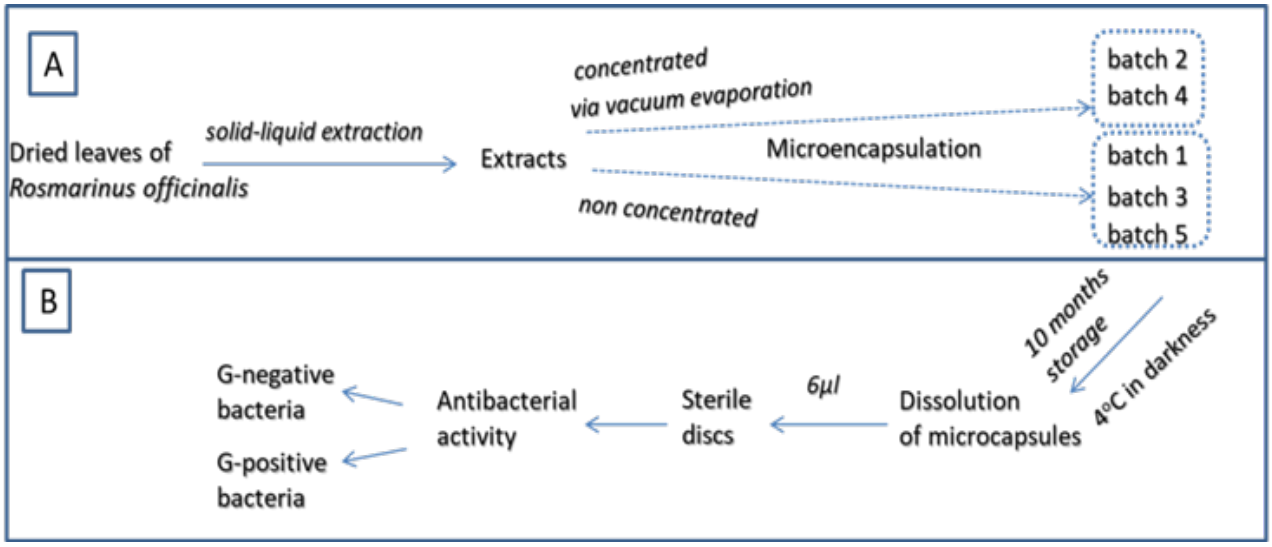

Fig. 1. Schematic representation of the preparation (A) and antibacterial test of microencapsulated rosemary extracts after long period of storage (B).

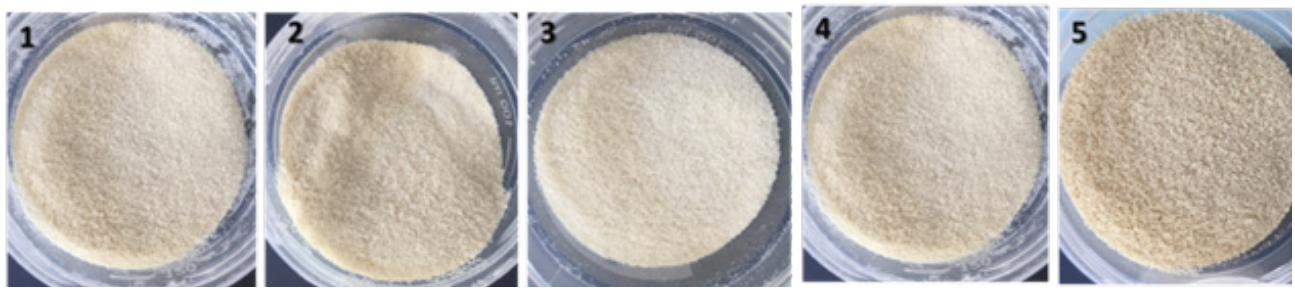

Fig. 2. Microencapsulated rosemary hydro-alcoholic extracts: batch 1 - (1), batch 2 - (2), batch 3 - (3), batch 4 - (4), batch 5 - (5).

broth and incubated overnight (24 h) at 37°C and 30°C for *E. coli* K12 and *B. subtilis* 356 respectively with shaking at 220 rpm. 100 µl of bacterial suspensions with concentration of 1×10^7 cfu/ml for the Gram-negative bacteria and 1×10^6 cfu/ml for the Gram-positive were seeded on agar plates with solid medium- LB or NB respectively by the pour plate technique. Sterile paper discs were impregnated with 6 µl of the 10 mass % water solution of each batch of microencapsulated rosemary extracts, and placed on the surface of the agar plate. A disc with the same amount of distilled water was used as a control. The formation of a clear zone (restricted bacterial growth) is an indication of antibacterial activity for the obtained materials. Inhibition zones were measured edge to edge across the zone of inhibition over the center of the disk according to the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol after incubation overnight at the preferred temperature for each strain [18]. Mean values of the inhibition zones were determined by performing the experiments in triplicate as described previously [19].

RESULTS AND DISCUSSION

We have tested the antibacterial activity of five batches of rosemary hydro-alcoholic extracts after 10 months storage against two bacterial strains. Batch 1 and batch 3 contain equal amount of non-concentrated rosemary extract while batch 2 and batch 4 contain the same amount of concentrated extract. The extract for batch 5 is also non-concentrated but the quantity of the impregnated material is three times larger in comparison to the other four samples. Another difference in the components is that the batch 1 and batch 2 contain an equal amount of maltodextrin and modified starch and in the case of batch 3, 4 and 5 only maltodextrin is used as an inert material [17].

The results from the antibacterial assay against *E. coli* K12 are presented in Fig. 3. For all tested samples together with the control one no inhibition zones were observed against the Gram-negative strain whereas prior to their storage an antibacterial activity was achieved [20]. Therefore it can be suggested that the antibacterial activity of the tested microencapsulated rosemary extracts was affected negatively by the 10 months storage.

Similar investigation with different type of plant extract and encapsulation technique was conducted by Wu et al. 2015. They have tested the antimicrobial stability of gelatin films loaded with cinnamon essential oil nanoliposomes. Disk diffusion method was used in the third and thirtieth days against *E. coli*, *Staphylococcus aureus* and *Aspergillus niger*. They also found decrease in the antimicrobial activity in all of the samples with time [21].

We have also investigated the antibacterial effect of the microencapsulated rosemary extracts against the Gram-positive bacteria *B. subtilis* 356. All five batches were investigated by the agar diffusion test as described in the experimental part and the results are summarized in Fig. 4. It illustrates that all the batches have antibacterial activity against the *B. subtilis* 356. Absence of inhibition zone is evident only for the control sample.

The smallest inhibition zones against the Gram-positive bacteria were observed in batch 1 and batch 3 (6.90 and 7.07 mm) which contain non concentrated rosemary extracts (Fig. 5). Larger inhibition zones were measured for batch 2 and 4 (impregnated with concentrated extract) with values of 7.23 mm and 7.50 mm, respectively. The highest antibacterial activity against *B. subtilis* 356 was achieved by batch 5 with a result-

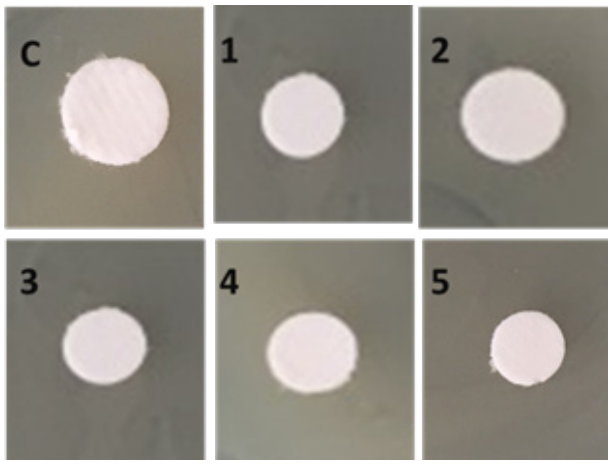

Fig. 3. Antibacterial test results of hydro-alcoholic microencapsulated rosemary extracts against *E. coli* K12: control - (C), batch 1 - (1), batch 2 - (2), batch 3 - (3), batch 4 - (4), batch 5 - (5).

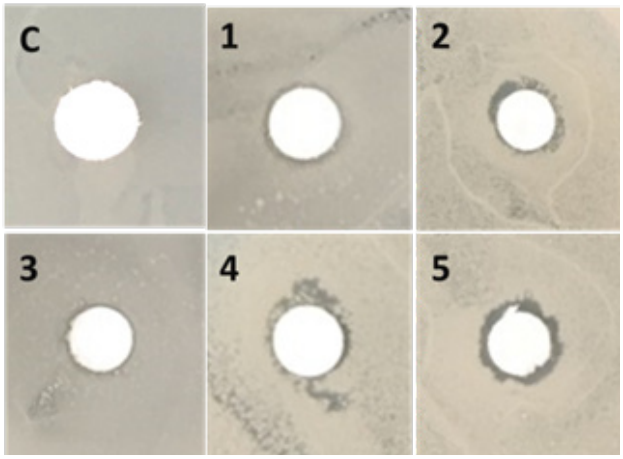

Fig. 4. Antibacterial test results of hydro-alcoholic microencapsulated rosemary extracts against *B. subtilis* 356: control - (C), batch 1 - (1), batch 2 - (2), batch 3 - (3), batch 4 - (4), batch 5 - (5).

ing zone of 8.00 mm. These microcapsules contain the higher quantity of rosemary extract in the microcapsules, thus demonstrating a stronger antibacterial activity of the sample.

In their work Donsì et al., 2011, investigated the effect of encapsulation on the antimicrobial activity of terpenes mixture. At the same time they observed that the minimal inhibitory concentration (MIC) of the terpenes mixture prior and after encapsulation depends on the microorganism tested. For the bacteria *E. coli* they have reported reduction in the MIC of the encapsulated terpenes mixture while for the *Lactobacillus delbrueckii* (Gram- positive bacteria) the nanoencapsulation caused no reduction and even increasing of the MIC for some of the samples [16].

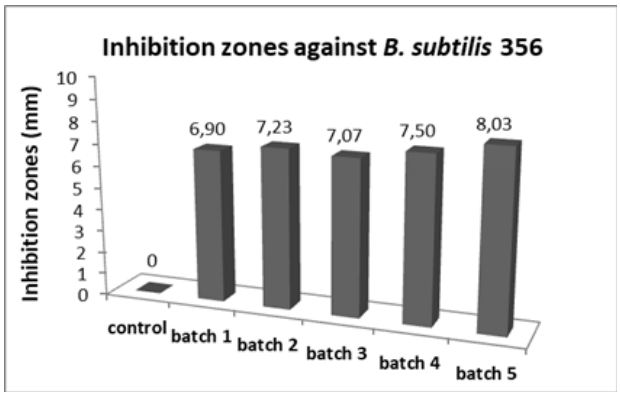

Fig. 5. Inhibition zones of hydro-alcoholic microencapsulated rosemary extracts against *B. subtilis* 356: control, batch 1, batch 2, batch 3, batch 4 and batch 5.

CONCLUSIONS

Agar diffusion test was used to analyze the antibacterial activity of microcapsules impregnated with rosemary extracts after long term storage. As opposed to previous tests using fresh microcapsules, no inhibition zones were observed against the Gram-negative bacteria *E. coli* K12 showing decrease in their activity in their activity. The microcapsules were also tested against the Gram-positive bacteria *B. subtilis* 356. In this case inhibition zones were observed for all of the batches. The results indicate that the long-term storage affected the activity against the Gram-negative strain. The results obtained with *B. subtilis* 356 demonstrate that even after 10 months storage in dark and cool place the microencapsulated rosemary extracts remain active.

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